Exhibit B

Int. J. Cancer: 87, 382–390 (2000)

Publication of the International Union Against Cancer

PHARMACOKINETICS AND MICRODISTRIBUTION OF POLYETHYLENE GLYCOL-MODIFIED HUMANIZED A33 ANTIBODY TARGETING COLON CANCER XENOGRAFTS

P. Markus DECKERT[1], Achim JUNGBLUTH[1], Nicholas MONTALTO[1], Mike A. CLARK[2], Ronald D. FINN[3], Clarence WILLIAMS JR.[1], Elizabeth C. RICHARDS[1], Katherine S. PANAGEAS[4], Lloyd J. OLD[1], and Sydney WELT[1]*

[1]*Ludwig Institute for Cancer Research, New York, New York, USA*
[2]*Department of Biology, University of Kentucky, Lexington, Kentucky, USA*
[3]*Medical Physics, Memorial Sloan-Kettering Cancer Center, New York, New York, USA*
[4]*Biostatistics, Memorial Sloan-Kettering Cancer Center, New York, New York, USA*

Therapeutic proteins have been conjugated with polyethylene glycol (PEGylation) to reduce immunogenicity and enhance circulating dose. Here we have investigated the effect of PEGylation on immunogenicity, pharmacokinetics, and histologic microdistribution of tumor-targeting antibodies with humanized A33 antibody (huA33) as a model system. Conjugation of huA33 with methoxy-PEG of M_r 5,000 (32%–34% of primary amines modified) or M_r 20,000 (16%–18% modification) preserved >50% of native huA33 binding to SW1222 colon cancer cells. In mice, both PEGylated forms cleared from serum moderately slower than native huA33. After repeated immunization with PEG-huA33, anti-antibody titers in immunocompetent mice were <5% of those in huA33-treated controls. Both PEG-huA33 forms reached approx. 75% of the maximum tumor dose of huA33 in SW1222-xenografted mice, but their tumor:blood ratios were considerably reduced. To demonstrate immunologic specificity of PEG-huA33 targeting in SW1222 tumor-bearing mice, antigenic sites were presaturated by injecting excess native huA33. This reduced subsequent uptake of PEG-huA33 by up to 80%, whereas presaturation with hu3S193 control antibody had no significant effect. To assess the microdistribution of antibody uptake in the same xenograft model, tumor tissue resected at different time points after antibody administration was examined for human IgG by immunohistochemistry. Both PEG preparations achieved the same peak staining intensity and homogeneity as native huA33 with a delay of several hours. Given the measured reduction in immunoreactivity *in vitro*, these results demonstrate that the tumor targeting potential of huA33 in vivo is preserved at PEGylation levels sufficient to suppress immunogenicity. *Int. J. Cancer* 87:382–390, 2000.
© *2000 Wiley-Liss, Inc.*

Induction of immune reactions is a major obstacle to repeated clinical administration of antibodies. Chimeric or humanized mouse monoclonal antibodies (MAbs) (King et al., 1995) and antibodies derived from human DNA libraries by phage display (Hoogenboom et al., 1992) have been developed to overcome this limitation. However, even fully humanized antibodies have been found to elicit human-anti-human immune responses (Welt et al., 1997). Furthermore, humanization may not be feasible with fusion proteins of antibodies and heterologous effector proteins. First, many of these effector proteins are of non-human origin and have no human counterpart, such as bacterial toxins (Reiter and Pastan, 1998) and prodrug-activating enzymes (Melton and Sherwood, 1996). Second, even if the components of these constructs were human-derived or fully humanized, their junction region may still represent immunogenic epitopes.

Conjugation of therapeutic drugs with poly[ethylene glycol] (PEG) has been successfully employed to increase their circulating half-life and solubility as well as to reduce immunogenicity and toxicity. This approach has been applied clinically, allowing bacterial enzymes such as L-asparaginase to be administered repeatedly even in patients who had previously displayed hypersensitivity to the foreign protein (Ettinger et al., 1995).

While increased serum half-life and reduced immunogenicity are widely accepted as general effects of PEGylation on drug molecules, its role in tumor targeting is less clear. Several groups have reported increased passive tumor uptake of liposomes, cytokines (reviewed in Francis et al., 1996), and non-peptide drugs (Senter et al., 1995; Westerman et al., 1998) and suggested a potential role for PEG in increasing passive targeting. This effect has been proposed to be due to the leakiness of tumor neovasculature (Jain, 1990), facilitating extravasation into tumor but not into normal tissue with intact vasculature. However, tumor:blood ratios were markedly reduced compared with the non-PEGylated product, and in one study that has investigated localization histologically, the drug was predominantly found in or around tumor vasculature (Westerman et al., 1998).

In active targeting, i.e., tumor uptake due to specific binding, preclinical studies of PEG-conjugation have mainly focused on Fab′ and $F(ab)_2$ antibody fragments. The effect of PEGylation on tumor localization appeared to depend on the protein size: While tumor localization of PEGylated complete IgG was reduced compared with the native antibody (Kitamura et al., 1991), several groups have reported increased absolute tumor uptake of Fab′ or $F(ab)_2$ fragments (Delgado et al., 1996; Eno-Amooquaye et al., 1996; Kitamura et al., 1991). As in passive targeting, however, reduced tumor:blood ratios were observed after PEGylation of Fab′ or $F(ab)_2$ fragments (Delgado et al., 1996; Eno-Amooquaye et al., 1996). Two factors are thought to be responsible for this effect: reduced clearance rate and reduced diffusion of macromolecules. Diffusion characteristics have been investigated in detail for non-modified antibodies and $F(ab)_2$ fragments, and several authors have concluded that tumor-directed macromolecules in general will not be able to achieve homogeneous distribution in tumor tissue due to elevated convective intratumoral pressure and low diffusion capacity of macromolecules (Francis et al., 1996; Jain, 1990; van Osdol et al., 1991). Together, these observations raise the question whether active targeting is feasible with PEG-conjugated antibodies, or if the previously described increase in

Abbreviations: **Ab**, antibody; **Fab′**, **$F(ab)_2$**, antibody fragments comprising one or two antigen-binding domains, respectively; **CDR**, complementarity-determining region; **huA33**, **hu3S193**, humanized A33 and 3S193 antibodies, repectively; **PBS**, phosphate buffered sodium chloride solution; **PEG 5**, **PEG 12**, and **PEG 20**, methoxy-polyethylene glycol succinimidyl-succinate of M_r 5000, M_r 12,000, and M_r 20,000, respectively.

Grant sponsor: National Cancer Institute; Grant numbers: CA-33049, CA-08748; Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: De602/1-1 (P.M.D.).

*Correspondence to: Sydney Welt, Ludwig Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue #813K, New York, NY 10021, USA. Fax: +1 (212) 717 3100.
E-mail: welts@mskcc.org

Received 23 October 1999; Revised 19 January 2000; Accepted 20 January 2000

tumor localization is a result of nonspecific accumulation in the interstitial space surrounding tumor vasculature.

MAb A33 recognizes a newly characterized cell-surface differentiation antigen of approximately 43 kDa molecular weight that belongs to the immunoglobulin superfamily. It is expressed on normal human gastrointestinal epithelium and on approx. 95% of primary or metastatic colon cancers but is absent in most other normal tissues (Heath et al., 1997). Some colon cancer cell lines, such as SW1222, express large amounts of the A33 antigen, binding up to 800,000 antibody molecules per cell. Upon binding to the A33 antigen, mAb A33 is internalized into a yet incompletely characterized vesicular compartment, and a significant fraction of the internalized antibody is recycled back to the cell surface (Daghighian et al., 1996). The A33 antigenic system has been the focus of several clinical studies in patients with colon cancer. Phase I/II clinical trials have shown that murine mAb A33 (i) localizes with high specificity to colon cancer tissue; (ii) is retained for prolonged periods of up to 6 weeks in the cancer but clears within 5 to 6 days from normal colon; and (iii) has anti-tumor activity as a carrier of ^{125}I or ^{131}I (Welt et al., 1994, 1996). A humanized version of the A33 antibody (huA33) has been constructed (King et al., 1995) and is currently being evaluated in clinical trials (Welt et al., 1997).

In this study we investigate the effect of PEGylation on tumor targeting and immunogenicity of huA33 in an established mouse xenograft model (Barendswaard et al., 1998).

MATERIAL AND METHODS

Animals and cell lines

Eight-week-old female outbred CD-1 mice (Charles River Laboratories, Wilmington, MA) and 8-week-old female athymic NCr-*nu*BR mice (nude mice; Taconic, Germantown, NY) were maintained at the Memorial Sloan-Kettering Cancer Center (MSKCC) Research Animal Resource Center. All animal experiments were performed under protocol 90-07-016, approved by the MSKCC Institutional Animal Care and Use Committee.

The human colon carcinoma cell lines SW1222 and HCT15 were from the cell bank of the Ludwig Institute for Cancer Research at MSKCC. Cells were maintained at 37°C and 5% CO_2 in Eagle's minimum essential medium supplemented with 1% (v/v) non-essential amino acids and 10% (v/v) FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and harvested using 0.1% (v/v) trypsin and 0.02% (v/v) EDTA (all reagents: GIBCO, Grand Island, NY).

SW1222 xenografts in nude mice

Nude mice were injected with 10^7 washed SW1222 cells in 150 μl sterile buffer (0.15 M NaCl and 0.1 M sodium phosphate, pH 7.4) into the left thigh muscle. Subsequent experiments were conducted when the tumor mass had reached a diameter of 0.4 to 0.6 cm, corresponding to a weight of 350 to 400 mg.

PEG modification of humanized antibodies

Methoxy-PEG-succinimidyl-succinate of M_r 5,000, 12,000, or 20,000 (Shearwater Polymers, Huntsville, AL) was weighed directly into the reaction tube, and 10 mg humanized A33 (King et al., 1995), or, for control experiments, 10 mg hu3S193 (Kitamura et al., 1994) were added in 10 ml of 100 mM sodium phosphate buffer, pH 7.4. The mixture was immediately vortexed vigorously for 30 sec and allowed to react for 60 min at room temperature under moderate shaking. Unreacted PEG was removed by ultrafiltration with a cutoff of 50 kDa (Ultrafree cartridges, Millipore, Marlborough, MA). The protein concentration was adjusted to 1.0 mg/ml, and purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 6% or 4%–12% precast gels and Coomassie staining (Novex, San Diego, CA).

Determination of modified primary amines. Unreacted primary amines were detected by mixing 150 μl of the purified conjugate with 50 μl of 1 mg/ml fluorescamine (Sigma, St. Louis, MO) in acetone and measuring fluorescence at an excitation wavelength of 360 nm ± 20 and an emittance wavelength of 460 nm ± 20 (modified after Stocks et al., 1986). The proportion of modified primary amines was calculated based on native (unconjugated) antibody as a standard.

Antibody binding activity (mixed hemadsorption assay). Binding of immunoglobulin to SW1222 tumor cells was detected by erythrocyte-bound protein A as previously described (Welt et al., 1994). The antibody binding titer was defined as the highest dilution that produced unequivocal rosetting of erythrocytes on tumor cells.

Radiolabelling of huA33 and hu3S193 antibodies

In the context of clinical imaging and therapeutic studies of radioiodinated A33, iodine 131 was selected for radiolabelling. Native and PEG-conjugated antibodies were labelled and assayed as described previously (Welt et al., 1994), using the chloramine T method. One milligram of the antibody was mixed with sodium iodide 131 (^{131}I) (1 mg/74 MBq) in 400 μl chloramine T solution (2 mg/ml). The reaction was terminated with 400 μl sodium metabisulfite (10 mg/ml), and the product was purified using a sterile Sephadex G25 column (Pharmacia) that had been preconditioned with sterile saline solution containing 5% human serum albumin. The MAb fractions were pooled and passed through a 0.2-μm filter. The specific activity was 7.4 to 9.3 MBq/mg. Each freshly prepared sample was tested for radiochemical purity by radio-thin-layer chromatography, for binding of ^{131}I to the antibody by trichloroacetic acid precipitation (>95% counts bound) and for immunoreativity by absorption of 0.1 μg/ml ^{131}I-antibody with sequential tubes containing 2×10^7 antigen-positive cells (Welt et al., 1994). Background activity was determined by pretreating cell pellets with a >100-fold excess of unlabelled antibody prior to adding radiolabelled antibody. Immunoreactivity was calculated by subtracting background radioactivity (counts per minute, CPM) from cell-bound ^{131}I radioactivity after washing twice in PBS and dividing the remaining fraction by the total CPM added. Immunoreactivity measured by this method was 50%-70% for huA33 and 34% for hu3S193.

Determination of antibody immunogenicity in mice

Groups of 5 CD-1 mice received 4 weekly I.V. injections of 25 μg native or PEG-modified huA33 antibody equalized for pure protein concentration (days 1, 8, 15, and 22). Blood samples were obtained before the first injection and after 5, 9, and 13 weeks.

Immunoreactivity against A33 was assessed by ELISA. Microtiter plates coated with 10 μg/well of huA33 and blocked with 1% w/v bovine serum albumin were incubated with mouse blood samples in duplicate dilution series. Bound murine antiserum was detected photometrically after incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG,A,M serum (Sigma, St. Louis, MO) and subsequent chromogenic reaction as the highest dilution that produced an absorbance greater than twice the background.

Tissue dosimetry of [^{131}I]huA33 in mice

Tumor xenograft-bearing nude mice and naive controls were injected I.V. with a single protein-equalized dose of 5 μg (approx. 10 μCi) radiolabelled native or PEGylated antibody in 100 μl sterile buffer (0.15 M NaCl, 0.1 M sodium phosphate, pH 7.2). For circulation clearance studies, blood samples of 10 to 30 μl were taken repeatedly at the indicated time points (see Results) from the retro-orbital plexus opposite of the injection site. To study organ distribution and tumor uptake, mice were sacrificed and blood, lung, liver, spleen, kidney, and tumor samples were obtained and weighed. The radioactive dose was measured by an automated gamma counter (model 1282 CompuGamma CS, LKB Wallac, Gaithersburg, MD) and compared with an aliquot of the injected preparation as a standard. Relative in vivo doses, expressed as percent of the injected dose per gram of tissue, were calculated as

% injected dose/g
= 100 × (sample CPM ÷ sample mass [g]) ÷ injected CPM.

Morphological studies

SW1222 tumor-bearing nude mice were treated as described above with native or PEG-conjugated huA33 or 3S193 antibody as indicated (see Results) and sacrificed 1, 4, 24, and 72 hr after injection. Tissues were harvested immediately after death, embedded in cryomolds filled with OCT compound (Tissue-Tek, Torrance, CA), and snap-frozen in dry ice-precooled isopentane. The frozen blocks were stored at −75°C. Cryostat sections (5 μm) were adhered to slides (Superfrost, Fisher Scientific, Pittsburgh, PA) and dried at room temperature for 30 min. Fixation was performed with acetone (4°C) for 10 min immediately before immunostaining. Immunohistochemistry was performed with an avidin-biotin system (ABC, Vector Laboratories, Burlington, CA). As primary reagent, a goat anti-human antibody (1:100; Jackson Laboratories, West Grove, PA) was applied at 4°C overnight followed by a biotinylated horse anti-goat secondary antibody (1:200, Jackson Laboratories). A control slide without the goat anti-human antibody incubation was included for all assays. For visualization, the chromogen 3,3′diaminobenzidine (DAB, Bio-Genex, San Ramon, CA) was used. Endogenous peroxidase was suppressed with 1% H_2O_2 for 30 min prior to application of the avidin-biotin complex. The slides were counterstained with Meyer's hematoxylin (Sigma) and dehydrated. As a staining control, one slide derived from a control animal treated only with buffer solution was stained directly with huA33, followed by the detection system described.

The slides were evaluated by a histopathologist, and the extent of staining was assessed semiquantitatively by visually estimating the proportion of positive tumor tissue in 25% increments as follows: −, no staining of tumor cells; +, <25% of tumor cells stained; ++, 25%–50%; +++, 50%–75%; and ++++, >75% of tumor cells stained.

Statistical analysis

Due to the small sample sizes, a permutation test was used to compare groups over time whereby random reordering of observations determined the significance level of a test. The test statistic used to perform this comparison was the difference in means summed over time. If the observed difference was extreme relative to the null permutation distribution, we could conclude that a statistically significant difference exists between the groups in the experiment.

RESULTS

Chemical characterization and immunoreactivity of PEG-huA33

In optimizing the conjugation process, we sought the highest PEG:Ab ratio for each PEG size that would not diminish antibody binding by more than 50% (one titration step). PEG 5, PEG 12, and PEG 20 were examined in PEG:Ab reactant ratios from 5 to 100. The reaction products were heterogeneous in size, reflecting different conjugation ratios achieved in the reaction. As PEG migrates slower than proteins of the same mass in polyacrylamide gel (Francis et al., 1996), electrophoresis could not reveal the actual m.w., but was used only to estimate the amount of unreacted antibody and to compare different PEG preparations. This showed that the result was dependent on the PEG size (Fig. 1A) as well as the molar reactant ratio (Fig. 1B). The actual percentage of modified primary amines was quantified using the fluorescamine assay (Stocks et al., 1986), which demonstrated almost complete reaction of PEG in PEG:Ab ratios of up to 70:1, resulting in an effective conjugation ratio close to the PEG:Ab ratio in the reaction (data not shown).

The immunoreactivity of PEG-Ab conjugates was determined by mixed hemadsorption assay on A33-antigen-positive SW1222 colon cancer cells. With PEG:Ab reactand ratios of up to 30:1 for PEG 5 and up to 15:1 for PEG 12 and PEG 20, no inhibition of antibody binding was observed that exceeded the limit of one titration step (Fig. 2), and these reactand ratios were selected for subsequent experiments. The corresponding conjugation results according to the fluorescamine assay were 32%–34% modified primary amines (average molar ratio, 28–30 PEG/antibody) for PEG 5 and 16%–18% for PEG 20 (molar ratio, 14–16 PEG/antibody; the apparent excess over the reactand ratio is within the error of the assay). At 4°C the conjugates were stable for at least 8 weeks according to gel electrophoresis and activity tests (data not shown).

PEG modification reduces immunogenicity of huA33 in mice

Immunocompetent CD-1 mice were immunized 4 times with 25 μg (1.25 mg/kg) of native, PEG 5-conjugated, or PEG 20-conjugated huA33, and anti-huA33 antibody titers were determined by ELISA. Mice that had received native huA33 produced increased

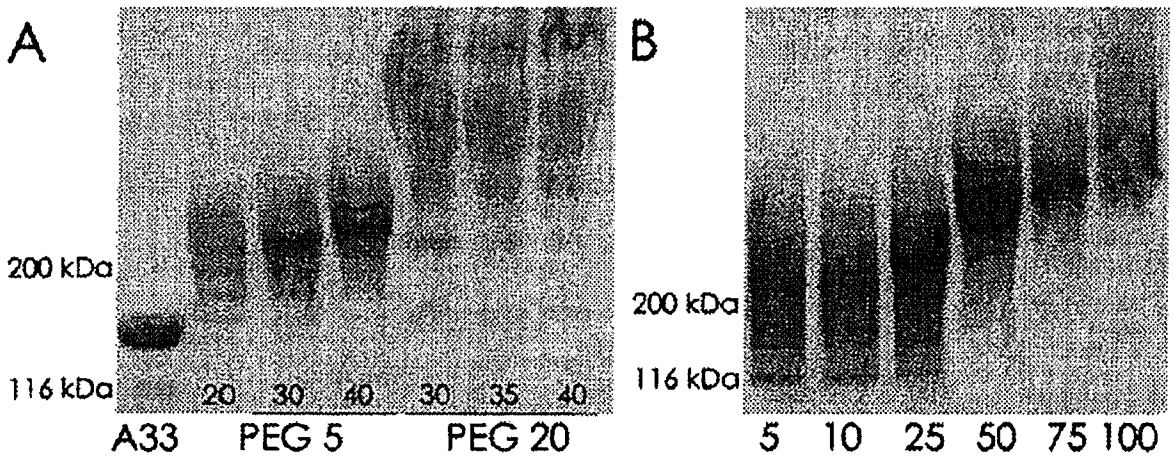

FIGURE 1 – PEG-huA33 conjugation results with different PEG sizes and molar ratios: huA33 conjugated with PEG as described in the text was run on 6% (A) or 4%–12% gradient (B) tris-glycine gels under nonreducing conditions. (A) huA33 conjugates with PEG 5 or PEG 20 in molar PEG:Ab reactant ratios as indicated. First lane (A33), native huA33 control. (B) Conjugates with PEG 12 in molar PEG:Ab ratios from 5 to 100.

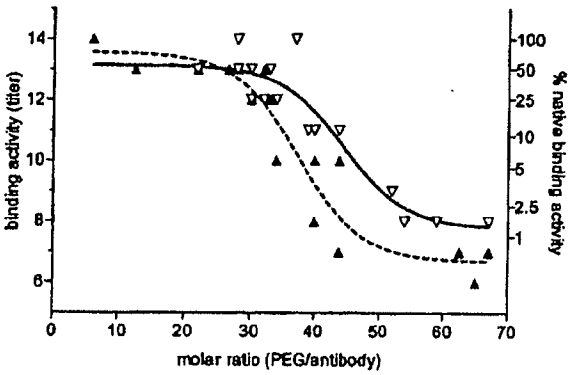

FIGURE 2 – Molar PEG:Ab ratio and binding activity: huA33 modified with either PEG 5 (▽—) or PEG 20 (▲- - -) at the indicated molar ratios as measured by fluorescamine assay was tested for binding to A33 antigen-positive SW1222 colon cancer cells. Average PEG ratio of the conjugate was measured by fluorescamine assay and antibody binding titer determined by mixed hemadsorption assay as described in the text. From these results, molar ratios of 30:1 (PEG 5) and 15:1 (PEG 20) were selected for subsequent experiments. Left-hand scale, dilution titer; right-hand scale, percent of activity (unmodified huA33 100%).

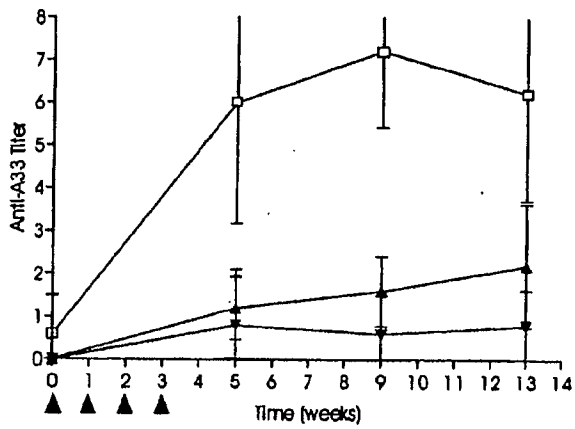

FIGURE 3 – Immunogenicity of huA33 and PEG-huA33. Groups of 5 mice each were injected with a protein-equalized dose of 5 μg of either native (□), PEG 5-conjugated (▼), or PEG 20-conjugated (▲) huA33 preparations at the indicated time points (arrows). Anti-huA33 binding activity was determined in an ELISA using unmodified huA33 as the target antigen (see text). Error bars indicate standard deviation. One-tailed paired *t*-test: native vs. PEG 5-huA33, P = 0.0217; native vs. PEG 20-huA33, P = 0.0211; PEG 5- vs. PEG 20-huA33, > 0.05.

antibody levels on day 35, and 4 of 5 mice reached a maximum titer of 256 on day 63 (Fig. 3). With both PEG preparations, however, a titer of 4 was not exceeded, and the highest titer was only observed on day 91 (significance levels over time, native huA33 vs. PEG 5-huA33, $P < 0.001$; vs. PEG 20-huA33, $P < 0.02$; no significant difference between PEG preparations).

To exclude immune reactions against epitopes formed by the PEG itself, we attempted to establish an ELISA with PEG-conjugated instead of native huA33. However, it was not possible to detect PEG-huA33 either directly or indirecuy using the above murine sera or anti-human control sera.

PEG-conjugation increases the circulating dose of humanized antibodies but reduces huA33 dose in tumor

The radioactive dose in blood was measured from 20 min to 7 days after injection of trace-labelled native or PEG-conjugated antibody preparations in non-tumor-bearing CD-1 mice (Fig. 4A). At the selected PEGylation ratios of 15:1 for PEG 20 and 30:1 for PEG 5, an increase in serum dose was observed compared with non-PEGylated A33 antibody, resulting in dose levels of PEGylated antibody that were 125% of the corresponding dose of native huA33 after 6 hr and 165% after 48 hr, converging thereafter (native huA33 vs. either PEG preparation, $P < 0.001$). Only when a considerably higher conjugation ratio, 40:1 with PEG 20, was used on the isotype control antibody 3S193 was a more marked increase in serum dose observed (Fig. 4B).

To determine tumor and organ uptake, groups of 5 SW1222 xenograft-bearing nude mice each were injected with 0.5 μg of trace-labelled native or PEGylated huA33. Animals from each group were sacrificed at 24, 48, 72, 96, and 168 hr post injection to measure blood, tumor, and organ doses, which were expressed as percent injected dose per milliliter of blood or gram of tissue, respectively. With all antibody preparations, the maximum tumor dose was reached at 24 hr, declining thereafter (Fig. 4C). Tumor uptake of PEGylated huA33 reached 73% to 82% of the uptake of native huA33 at corresponding time points (native huA33 vs. PEG 5, $P = 0.013$; vs. PEG 20, $P < 0.001$). Tumor:blood ratios also were significantly higher with native huA33 compared with either PEG-conjugate (PEG 5, $P = 0.001$, PEG 20, $P = 0.014$). At the last time point, the PEG 20-conjugate appears to reach a higher ratio than PEG 5-huA33, but over the time course no significant difference between PEG-conjugates was found (Fig. 4D).

In normal tissues, only small differences in uptake were observed between native and PEGylated huA33 (Fig. 5). While isolated significant differences could be calculated for kidney, spleen, and lung at the 96-hr time point, over time only in lung PEG 5-huA33 was retained at significantly higher levels than native huA33 ($p = 0.0316$).

In vivo tumor localization of ^{131}I-PEG-A33 is immunologically specific

In order to assess the immunologic specificity of antibody localization to tumor in vivo, the previous xenograft experiment was modified by pretreating mice with excess native antibody (unlabelled, non-PEGylated) to presaturate antigenic sites. Tumor-bearing nude mice were injected with 250 μg of either huA33 or hu3S193 control antibody. Six hours later, 5 μg of ^{131}I-labelled PEG 20-huA33 was injected, and animals were sacrificed after 21, 45, and 68 hr. No significant difference between the 2 pretreatment groups was observed for radioactive dose in blood, kidney, spleen, liver, or lung (data not shown). In tumor tissue, however, blocking with huA33 significantly reduced ^{131}I-PEG-A33 binding down to the levels of nonspecific binding in organ tissues ($P < 0.001$ for difference to unblocked control), whereas pretreatment with hu3S193 control antibody had no effect on PEG-huA33 binding (hu3S193-block vs. unblocked control, $P = 0.678$; vs. huA33-block, $P < 0.001$; Fig. 6).

PEG-A33 targets tumor cells with the same microdistribution pattern as unconjugated huA33

The results of the immunohistochemical staining are shown in Table 1 and Fig. 7. All tissues showed variable degrees of intravascular and stromal staining due to the presence of humanized antibody in blood vessels and connective tissue. No staining was present in the neoplastic xenograft tumor cells of the animals treated with hu3S193. Mice treated with native huA33 showed intense tumor cell staining at all time points. The largest difference in the extent of immunohistochemical staining was seen at 1 hr after injection. At this time point, only restricted distribution was visible with all 3 PEG conjugates of huA33, which showed homogeneous or almost homogeneous tumor localization from the 4-hr time point on.

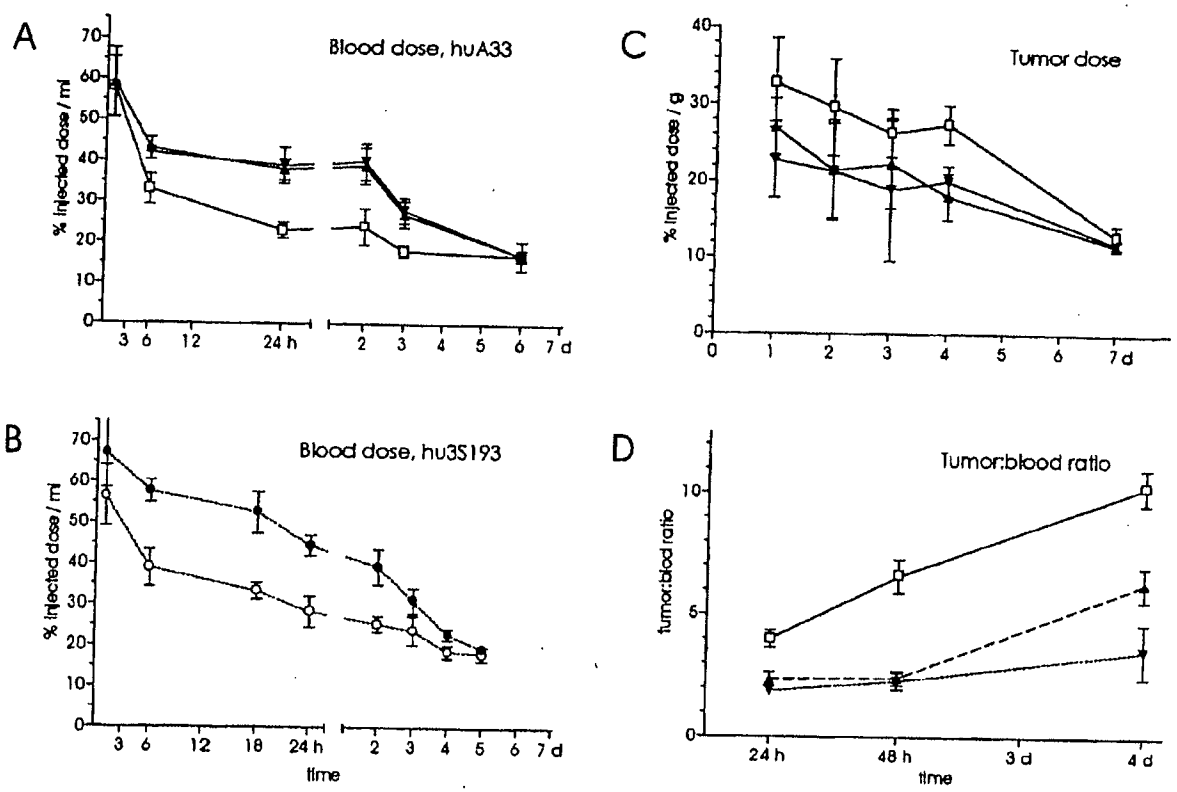

FIGURE 4 – Blood and tumor doses of native and PEG-modified antibodies in mice. Groups of 5 mice were injected with protein-equalized doses of 5 μg of one of the following ^{131}I-labelled antibody preparations: native (□), PEG 5-conjugated (▼), or PEG 20-conjugated (▲) huA33, or native (○) or PEG 20-conjugated (●) hu3S193. Blood and/or tumor tissue was obtained at the time points indicated, and radioactive dose per gram was measured and normalized for injected dose. (A, B) Elimination of native and PEGylated antibody preparations from the blood of non-tumor-bearing Swiss mice. (A) huA33; (B) hu3S193 control antibody. The PEG:Ab ratios were 15:1 for PEG 20-huA33, 30:1 for PEG 5-huA33, and 40:1 for PEG 20-3S193. Summarized data from 3 experiments. (C) Injected huA33-dose in tumors of nude mice bearing SW1222 xenografts of defined size. Mice were sacrificed at the time points indicated and the tumors resected. Summarized data from 2 experiments. (D) Tumor:blood ratios at indicated time points after injection of radiolabelled antibody into SW1222-tumor-bearing mice.

DISCUSSION

The A33 antigenic system has shown promising tumor-targeting in clinical trials (Welt et al., 1994, 1996). To reduce its immunogenicity, the A33 antibody has been fully humanized by CDR-grafting (King et al., 1995). However, in an ongoing clinical study, even this humanized version has induced immune reactions against the antibody in 4 of 11 patients (Welt et al., 1997). We have therefore explored PEG-conjugation as a means to overcome or reduce this limitation. This study demonstrates that PEG-conjugated huA33 antibody localizes to tumor tissue in vivo with immunological specificity. At conjugation ratios sufficient to suppress immunogenicity, PEG-huA33 showed homogeneous targeting to tumor tissue comparable to the native antibody. However, the proportional tumor dose of PEG-huA33 was reduced to approximately 75% of the dose achieved with the native antibody. Although elimination rates from tumor were similar for native and PEGylated antibody, tumor:blood ratios of the PEG conjugates were about one-third to one-half those of unmodified huA33, increasing over time for all 3 preparations as circulating antibody was eliminated from the vascular compartment.

In this study, we observed 3 phases in the micro-localization of non-PEGylated huA33: (i) initial targeting: as early as 1 hr post-injection, huA33 localized with high intensity to peripheral tumor cells; (ii) distribution in tumor tissue: staining throughout the tumor nodule was observed after approx. 4 hr; and (iii) clearance of nonspecific staining: stroma and vasculature were almost completely unstained after 24 hr, while tumor tissue remained homogeneously stained. With PEGylated huA33, the targeting process followed the same consecutive pattern with a delay of several hours.

Theoretical models have predicted that antibodies may not be able to achieve tumor targeting beyond the periphery of a tumor, as the inward directed concentration gradient would be insufficient to overcome the outward directed convective pressure gradient (Jain, 1990; van Osdol et al., 1991). However, this is not the case with the A33 antigenic system, since homogeneous distribution of A33 antibody throughout colon cancer tissue has been demonstrated in mice and humans (Barendswaard et al., 1998; Welt et al., 1994, 1996). The present study confirms these findings for PEG-conjugated huA33. A possible explanation for the fast and homogeneous distribution may be the high internalization rate of antigen-antibody complexes documented for A33 (Daghighian et al., 1996). The binding-site barrier model postulates that a high-affinity antibody to an abundantly expressed antigen will form a gradient from periphery to center, with most antibody binding at the entry site in the periphery, preventing further diffusion into tumor tissue (van Osdol et al., 1991). Therefore, one might

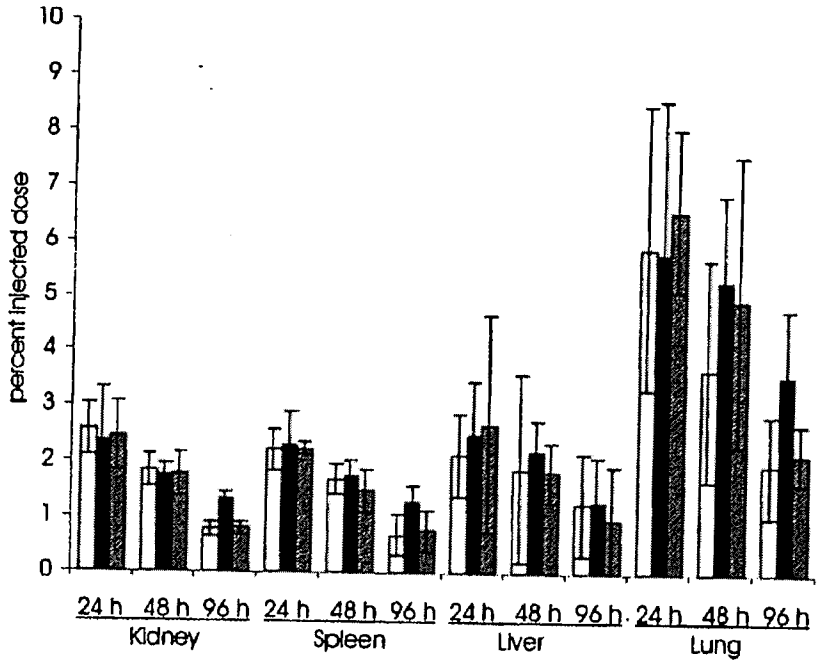

FIGURE 5 – Organ distribution of different huA33 preparations. Tumor-bearing nude mice were injected with a protein-equalized dose of 5 μg of ^{131}I-labelled native huA33 (white), PEG 5-conjugated huA33 (black), or PEG 20-conjugated huA33 (hatched). Percent injected dose per gram of tissue.

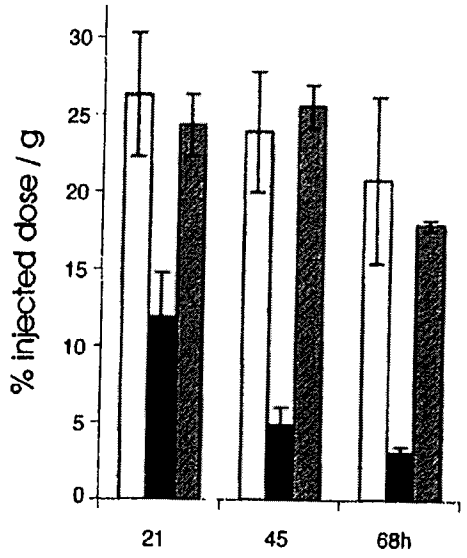

FIGURE 6 – Blocking of radiolabelled PEG 5-huA33 binding to tumor tissue by presaturation with unlabelled antibodies. Tumor-bearing nude mice were treated with unmodified huA33 (black) or control antibody hu3S193 (hatched) before being injected with ^{131}I-labelled PEG-huA33. Tumor doses were measured at the time points indicated and expressed as percent injected dose per gram of tissue. White: control without presaturation.

hypothesize that internalization of antigen-antibody complexes and the consequent depletion of antigenic binding sites would permit deeper penetration of antibody into the tumor. As long as a sufficient amount of antibody is present over time, antibody

TABLE I – SCORE OF IN VIVO STAINING OF SW1222 TUMOR XENOGRAFTS BY DIFFERENT ANTIBODY FORMULATIONS

	1 hr	4 hr	24 hr	72 hr
Buffer	–	–	–	–
Native hu3S193	–	–	–	–
Native huA33	++++	++++	++++	++++
PEG 5-huA33	+	++++	++++	+++
PEG 12-huA33	++	++++	++++	++++
PEG 20-huA33	++/+++	+++	++++	+++

For details on experiment and staining, see text. Slides from Fig. 7 were evaluated for distribution of tumor staining and assigned one of the following scores: completely negative (–), <25% (+), 25% to 50% (++), 50% to 75% (+++), and >75% (++++) of tumor cells stained.

localization would thus progressively advance towards the core of a tumor nodule.

Several authors have described increased passive, i.e., not antigen-specific, tumor targeting as an effect of PEGylation of various proteins and non-protein drugs (Francis et al., 1996; Senter et al., 1995; Westerman et al., 1998). To exclude that the tumor localization we observed represented mere passive uptake, we demonstrated antigen-specific binding of PEGylated huA33 in tumor-xenografted mice by presaturation of antigenic sites with unconjugated, unlabelled antibodies. Native huA33 reduced subsequent detection of radiolabelled PEG-huA33 to the level of nonspecific uptake in organ tissues, while pretreatment with a control antibody had no significant effect. In addition, tumor:blood and tumor:organ ratios were highest during the elimination phase of the antibody, which is consistent with a binding force that retained PEG-huA33 in tumor against a concentration gradient. These results allow the conclusion that PEG-huA33 targeting is immunologically specific and not due to nonspecific pharmacokinetic characteristics of a PEGylated protein.

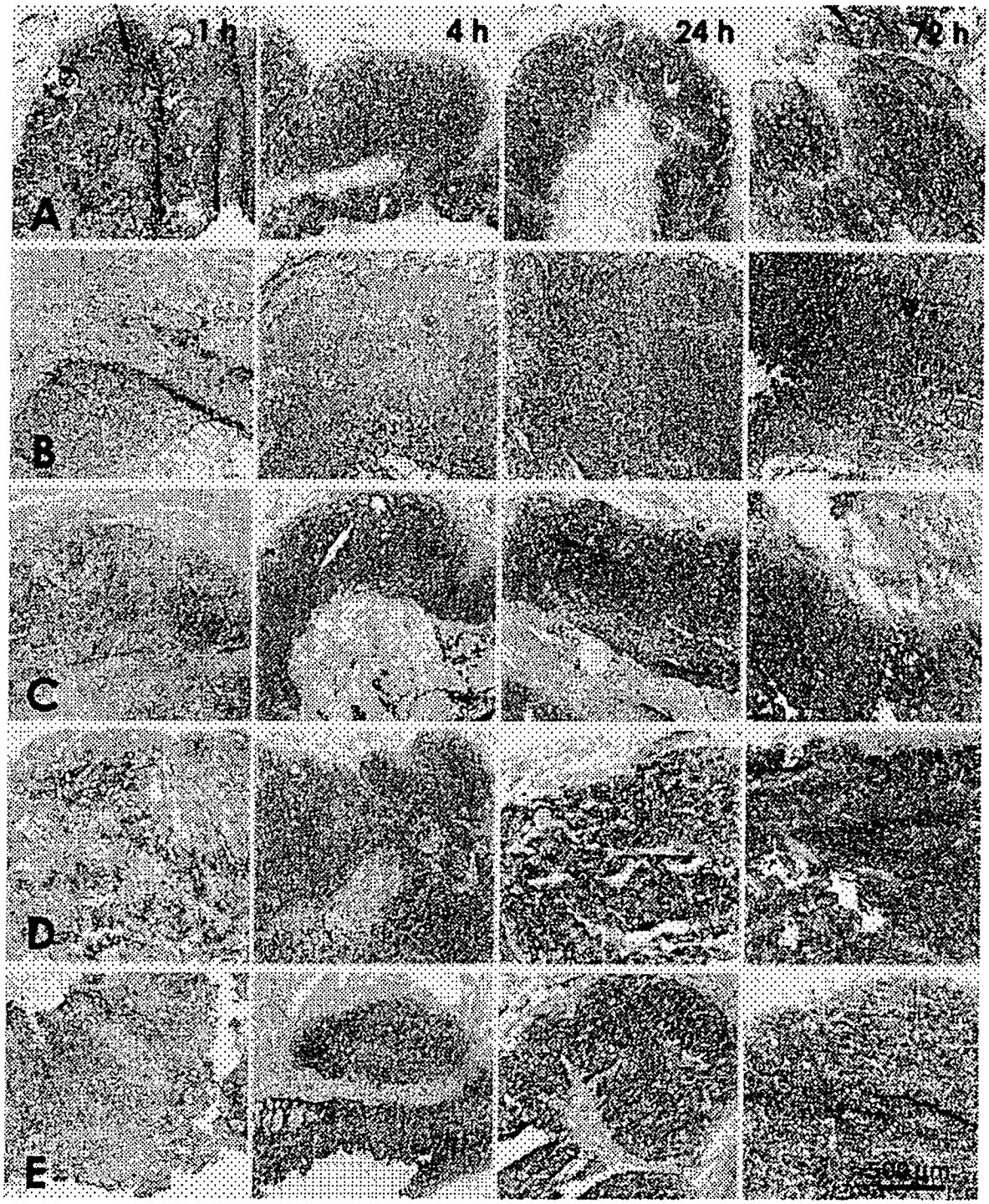

FIGURE 7 – Morphological localization of different antibody preparations in SW1222 colon xenografts in mice. Mice bearing tumors of defined size were injected with a protein-equalized dose of 5 µg of the antibody solutions listed below. At the time points indicated, tumors were resected, and thin sections were subsequently stained with IgG-specific goat anti-human primary and biotinylated horse anti-goat secondary antibodies and a streptavidin-alkaline phosphatase conjugate, which was detected by reaction with a chromogenic substrate. Representative fields. Antibodies: (A) native huA33; (B) hu3S193 control; (C) PEG 5-huA33; (D) PEG 12-huA33; (E) PEG 20-huA33.

The objective of PEG-conjugation in this study was to reduce the immunogenicity of a therapeutic antibody. Induction of antibodies against huA33 as a xenogenic protein in mice was reduced by more than 95% after modification of 32%–34% of primary amines with PEG 5 or of 16%–18% of primary amines with PEG 20.

Formally, our results do not exclude the possibility of antibodies against new epitopes formed by the introduction of PEG, as we only tested for murine antibodies against huA33, not PEG-huA33. However, it was not possible to establish an ELISA using PEG-huA33 as the target antigen. Probably this failure to detect PEG-huA33 immunologically reflects the same mechanisms that have been reported to reduce antigenicity of PEGylated proteins in vivo (Chaffee et al., 1992).

Using the sulfhydryl-methoxy-PEG method, we have optimized the conjugation conditions so as to achieve the highest possible PEGylation degree while leaving little or no antibody unconjugated and not incurring more than 50% (one titer step) loss in antibody binding activity. However, as we have shown that tumor uptake of PEG-huA33 is dependent on its antigen specificity, lower binding activity was likely to contribute to the observed reduction of tumor:blood ratios compared with native antibody. Reduced protein function is not necessarily an effect of the presence of PEG per se but may also be due to linker moieties and to harsh conditions during the conjugation reaction. These adverse effects may both be possible to eliminate by techniques such as the linker-less Tresyl-mPEG method (Francis et al., 1996).

Still, considering the distribution of primary amines as potential conjugation sites, impairment of immunoreactivity may be inevitable with all random linking methods. Of the 88 primary amines provided by lysine residues in each huA33 molecule, 20 are found in the variable regions. While only 4 of these are located in CDRs, close proximity of primary amines in the variable region framework will be likely to account for various degrees of steric hindrance in a sizeable proportion of PEGylated antibody molecules. New site-specific rather than random linking techniques (Lee et al., 1999), however, may lead to an improved balance of immunogenicity and function.

This being an exploratory study, it was not designed to determine conclusively the optimal combination of PEGylation degree and PEG type. However, conjugation with PEG 20 displayed a trend towards higher immunogenicity than PEG 5, but it also showed a trend towards higher tumor:blood ratios of the PEG 20 conjugate during the elimination phase. While these observations were not statistically significant, it seems plausible that both the reduction of immunogenicity and of antibody binding should depend more on the number of attached PEG molecules than on their size. Moreover, should this finding be reproducible, it would imply that the putatively better binding function of the PEG 20 conjugate may be sufficient to overcome the substantial diffusion obstacle constituted by the 2-fold higher total increase in molecular mass compared with the PEG 5 conjugate. Resolving these questions in a detailed comparative approach will be the objective of future studies using the improved conjugation methods mentioned above. Beyond comparing various PEGylation methods and PEG sizes, these studies also will have to question our preliminary assumption that the highest possible PEGylation degree be desirable. It might well be possible to achieve the same reduction in immunogenicity with lower PEGylation degrees, thus incurring less impairment of antibody function and tumor localization.

In apparent contradiction to the concept of PEGylation as a method of reducing immunogenicity, Brumeanu et al. (1995) used low-degree PEG conjugation (8% modification of primary amines) to enhance the immune response against viral epitopes represented by immunoglobulins, thus obviating the need for an adjuvant in a murine vaccination model. As this in effect is an anti-idiotypic immunization, the question arises if PEGylation may promote selection for responses against the antigen-binding regions of antibodies. It has been demonstrated in the clinic that antibodies recognizing a natural receptor are capable of eliciting anti-idiotypic anti-antibody responses, which then mimic features of the original receptor (Deckert et al., 1996). In the case of the A33 antigen, whose natural ligand is yet to be identified, such a mechanism could carry a potential for autoimmune reactions.

PEGylation caused a modest increase in the circulating dose of huA33 in comparison with the native antibody. A more marked increase in circulation time was observed only at PEGylation degrees that significantly reduced immunoreactivity. The immediate effects of PEG conjugation on circulating dose and tumor localization are determined mainly by 2 factors: protection from enzymatic degradation and reduced diffusion due to increased size (Francis et al., 1996). Protection from degradation should prolong the circulating half-life of a PEGylated molecule independent of its size. The increase in effective diameter, however, is a double-edged effect. On one hand, it prolongs circulating half-life, an effect most prominent with small proteins that pass the renal filter in their native form but are retained after PEGylation. This is the case with antibody fragments, which have shown a marked increase in serum half-life after PEGylation, whereas complete IgG antibodies pass the renal filter neither native nor PEGylated (Delgado et al., 1996; Eno-Amooquaye et al., 1996; Kitamura et al., 1991). On the other hand, an increase in diameter also impedes the diffusion of a protein of any size in perivascular space and tumor tissue, reducing its capability of tumor targeting and penetration. On balance, the effect of the increased diameter on tumor localization is more favorable for smaller antibody fragments, which are excreted rapidly in their non-PEGylated forms, while in larger molecules, such as complete IgG, the impeding effect on tumor targeting prevails.

Furthermore, additionally increasing the already long circulating half-life of native antibodies by PEGylation also reduces the tumor:blood ratio. While a pure immunotherapeutic approach might benefit from an increase in circulation time per se, this effect could considerably impair the efficacy of radioimmunotherapy or antibody-enzyme prodrug therapy. With these therapeutic approaches, a high antibody blood concentration is desirable during the initial phase of tumor penetration. Once the antibody has bound to tumor tissue, rapid clearance of circulating antibody would minimize unwanted systemic effects. Hence it has been suggested to employ clearing antibodies that neutralize the respective tumor-targeting antibody in order to accelerate elimination of unbound antibody from the vascular compartment (Eno-Amooquaye et al., 1996).

The A33 antibody has shown high selectivity for primary and metastatic colon tumor localizations in clinical phase I and phase II studies (Welt et al., 1994, 1996). As the current investigation is limited to an animal model, however, predictions regarding biodistribution in humans have to be made with caution. The human A33 antigen is also expressed in normal colon tissue (Heath et al. 1997; Welt et al., 1994), but the murine equivalent of the human A33 antigen is not recognized by the A33 antibody directed against the human antigen. Therefore, we could not study the effect of PEGylation on antibody localization to normal colon in the mouse model. Only clinical trials will be able to assess if the rapid clearance of A33 antibody from normal colon that has been demonstrated in the clinic will be maintained by a PEG-conjugated preparation.

REFERENCES

BARENDSWAARD, E.C., SCOTT, A.M., DIVGI, C.R., WILLIAMS, C.J., COPLAN, K., RIEDEL, E., YAO, T.J., GANSOW, O.A., FINN, R.D., LARSON, S.M., OLD, L.J. and WELT, S., Rapid and specific targeting of monoclonal antibody A33 to a colon cancer xenograft in nude mice. *Int. J. Oncol,* **12**, 45–53 (1998).

BRUMEANU, T.D., ZAGHOUANI, H., ELAHI, E., DAIAN, C. and BONA, C.A., Derivatization with monomethoxypolyethylene glycol of Igs expressing viral epitopes obviates adjuvant requirements. *J. Immunol.* **154**, 3088–3095 (1995).

CHAFFEE, S., MARY, A., STIEHM, E.R., GIRAULT, D., FISCHER, A. and HERSHFIELD, M.S., IgG antibody response to polyethylene glycol-modified adenosine deaminase in patients with adenosine deaminase deficiency. *J. Clin. Invest.,* **89**, 1643–1651 (1992).

DAGHIGHIAN, F., BARENDSWAARD, E., WELT, S., HUMM, J., SCOTT, A., WILLINGHAM, M.C., MCGUFFIE, E., OLD, L.J. and LARSON, S.M., Enhancement of radiation dose to the nucleus by vesicular internalization of iodine-125-labelled A33 monoclonal antibody. *J. Nucl. Med.,* **37**, 1052–1057 (1996).

DECKERT, P.M., BALLMAIER, M., LANG, S., DEICHER, H. and SCHEDEL, I., CD4-imitating human antibodies in HIV infection and anti-idiotypic vaccination. *J. Immunol.,* **156**, 826–833 (1996).

DELGADO, C., PEDLEY, R.B., HERRAEZ, A., BODEN, R., BODEN, J.A., KEEP, P.A., CHESTER, K.A., FISHER, D., BEGENT, R.H. and FRANCIS, G.E., Enhanced tumour specificity of an anti-carcinoembrionic antigen Fab' fragment by poly(ethylene glycol) (PEG) modification. *Brit. J. Cancer* **73**, 175–182 (1996).

ENO-AMOOQUAYE, E.A., SEARLE, F., BODEN, J.A., SHARMA, S.K. and BURKE, P.J., Altered biodistribution of an antibody-enzyme conjugate modified with polyethylene glycol. *Brit. J. Cancer* **73**, 1323–1327 (1996).

ETTINGER, L.J., KURTZBERG, J., VOUTE, P.A., JURGENS, H. and HALPERN, S.L., An open-label, multicenter study of polyethylene glycol-L-asparaginase for the treatment of acute lymphoblastic leukemia. *Cancer,* **75**, 1176–1181 (1995).

FRANCIS, G.E., DELGADO, C., FISHER, D., MALIK, F. and AGRAWAL, A.K., Polyethylene glycol modification: relevance of improved methodology to tumour targeting. *J. Drug Target.,* **3**, 321–340 (1996).

HEATH, J.K., WHITE, S.J., JOHNSTONE, C.N., CATIMEL, B., SIMPSON, R.J., MORITZ, R.L., TU, G.F., JI, H., WHITEHEAD, R.H., GROENEN, L.C., SCOTT, A.M., RITTER, G., COHEN, L., WELT, S., OLD, L.J., NICE, E.C. and BURGESS, A.W., The human A33 antigen is a transmembrane glycoprotein and a novel member of the immunoglobulin superfamily. *Proc. Nat. Acad. Sci. (Wash.),* **94**, 469–474 (1997).

HOOGENBOOM, H.R., MARKS, J.D., GRIFFITHS, A.D. and WINTER, G., Building antibodies from their genes. *Immunol. Rev.* **130**, 41–68 (1992).

JAIN, R.K., Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. *Cancer Res.,* **50**, 814s–819s (1990).

KING, D.J. and 19 OTHERS, Preparation and preclinical evaluation of humanised A33 immunoconjugates for radioimmunotherapy. *Brit. J. Cancer,* **72**, 1364–1372 (1995).

KITAMURA, K., STOCKERT, E., GARIN-CHESA, P., WELT, S., LLOYD, K.O., ARMOUR, K.L., WALLACE, T.P., HARRIS, W.J., CARR, F.J. and OLD, L.J., Specificity analysis of blood group Lewis-y (Le(y)) antibodies generated against synthetic and natural Le(y) determinants. *Proc. Nat. Acad. Sci. (Wash.),* **91**, 12957–12961 (1994).

KITAMURA, K., TAKAHASHI, T., YAMAGUCHI, T., NOGUCHI, A., TAKASHINA, K., TSURUMI, H., INAGAKE, M., TOYOKUNI, T. and HAKOMORI, S., Chemical engineering of the monoclonal antibody A7 by polyethylene glycol for targeting cancer chemotherapy. *Cancer Res.,* **51**, 4310–4315 (1991).

LEE, L.S., CONOVER, C., SHI, C., WHITLOW, M. and FILPULA, D., Prolonged circulating lives of single-chain Fv proteins conjugated with polyethylene glycol: a comparison of conjugation chemistries and compounds. *Bioconjug. Chem.,* **10**, 973–981 (1999).

MELTON, R.G. and SHERWOOD, R.F., Antibody-enzyme conjugates for cancer therapy. *J. Nat. Cancer Inst.* **88**, 153–165 (1996).

SENTER, P.D., SVENSSON, H.P., SCHREIBER, G.J., RODRIGUEZ, J.L. and VRUDHULA, V.M., Poly(ethylene glycol)-doxorubicin conjugates containing beta-lactamase-sensitive linkers. *Bioconjug. Chem.,* **6**, 389–394 (1995).

STOCKS, S.J., JONES, A.J., RAMEY, C.W. and BROOKS, D.E., A fluorometric assay of the degree of modification of protein primary amines with polyethylene glycol. *Anal. Biochem.,* **154**, 232–234 (1986).

VAN OSDOL, W., FUJIMORI, K. and WEINSTEIN, J.N., An analysis of monoclonal antibody distribution in microscopic tumor nodules: consequences of a "binding site barrier". *Cancer Res.* **51**, 4776–4784 (1991).

WELT, S., DIVGI, C.R., KEMENY, N., FINN, R.D., SCOTT, A.M., GRAHAM, M., GERMAIN, J.S., RICHARDS, E.C., LARSON, S.M., OETTGEN, H.F. and OLD, L.J., Phase I/II study of iodine 131-labelled monoclonal antibody A33 in patients with advanced colon cancer. *J. Clin. Oncol.* **12**, 1561–1571 (1994).

WELT, S., RITTER, G., COHEN, L., WILLIAMS, C.J., DANTIS, L., RICHARDS, E.C., KEMENY, N. and OLD, L.J., Phase I study of humanized A33 (huA33) antibody in patients with advanced colorectal cancer. *Proc. Annu. Meet. Amer. Soc. Clin. Oncol.* **16**, A1563 (1997).

WELT, S., SCOTT, A.M., DIVGI, C.R., KEMENY, N.E., FINN, R.D., DAGHIGHIAN, F., GERMAIN, J.S., RICHARDS, E.C., LARSON, S.M. and OLD, L.J., Phase I/II study of iodine 125-labelled monoclonal antibody A33 in patients with advanced colon cancer. *J. Clin. Oncol.* **14**, 1787–1797 (1996).

WESTERMAN, P., GLANZMANN, T., ANDREJEVIC, S., BRAICHOTTE, D.R., FORRER, M., WAGNIERES, G.A., MONNIER, P., VAN DEN BERGH, H., MACH, J.P. and FOLLI, S., Long circulating half-life and high tumor selectivity of the photosensitizer meta-tetrahydroxyphenylchlorin conjugated to polyethylene glycol in nude mice grafted with a human colon carcinoma. *Int. J. Cancer,* **76**, 842–850 (1998).